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DETERMINING PREGNANCY FROM BLUBBER IN THREE SPECIES OF DELPHINIDS

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ABSTRACT

We quantified progesterone in 110 blubber samples from dolphins of known reproductive status in order to test the accuracy of a method to determine pregnancy status in wild cetaceans. The samples were collected from fishery-bycaught delphinids of three species (*Delphinus delphis*, *Lissodelphis borealis*, and *Lagenorhynchus obliquidens*). We ascertained that blubber progesterone concentrations could clearly distinguish pregnant *D. delphis* (range 132–415 ng/g, mean 261 ng/g) from nonpregnant mature and immature ones (range 0.92–48.2 ng/g, mean 15.2 ng/g). We found similar dramatic differences in *L. borealis* and *L. obliquidens*. These results were insensitive to various blubber sampling depths and anatomical sampling locations on the body, suggesting relative homogeneity of progesterone levels throughout the blubber. However, no trend was found in blubber progesterone concentration with fetal length, indicating that although blubber progesterone appears to distinguish pregnancy status, it is unlikely to differentiate pregnancy stage. Based on the findings presented here we suggest that this method, when coupled with projectile biopsy procedures, can be used to assess the pregnancy status of free-ranging cetaceans and thus provide a new tool to determine pregnancy rates of wild populations.

Key words: blubber, adipose tissue, biopsy, progesterone, pregnancy, pregnancy rate, short-beaked common dolphin, *Delphinus delphis*, northern right-whale dolphin, *Lissodelphis borealis*, Pacific white-sided dolphin, *Lagenorhynchus obliquidens*, gestation, free-ranging.

Accurate, non-lethal approaches to determine cetacean reproductive status are needed to estimate pregnancy rate, an index of recruitment, for wild populations. In the past, reproductive status has been determined by examining the reproductive tracts of dead animals, and thus pregnancy-rate estimates have been made only from individuals collected from strandings, harvests, or incidental bycatch (Calzada *et al*.

1996, Heise 1997, Kasuya *et al*. 1997). There are at least two drawbacks to these approaches. First, these sample sets are frequently biased, with one or more demographic groups either over- or under-represented (George *et al*. 1995, Iwasaki and Kasuya 1997). The sources of these biases are generally not understood and as such, mathematical corrections cannot be made to obtain the true rates of pregnancy. Second, these approaches are restricted to opportunistic sampling; harvesting activities or the conditions that create strandings dictate the timing, location, and composition of the sample sets (Read 1990, Zeh *et al*. 1995, Hohn *et al*. 1996).

At least two non-lethal approaches to determine pregnancy have been previously employed. Perryman and Lynn (2002) used allometric measurements made from aerial photographs to accurately differentiate pregnancy status in gray whales. However, this methodology is effective only for mid- to late-term pregnancies when the girth-to-length ratio of pregnant females is much greater than non-pregnant ones. Consequently, data derived from this approach are easily interpreted only in populations with strong reproductive seasonality, from which photographs can be taken immediately prior to calving season. Rolland *et al*. (2005) showed that progestins in fecal material can differentiate pregnant right whales from non-pregnant ones. Though this appears to be an effective method, in most field situations fecal collection is impractical; opportunities to collect samples are rare (exceedingly so for many smaller cetaceans) and assessing whether there are multiple contributors of a single sample or repeat samplings of single individuals is challenging.

Other non-lethal methods (*i.e.*, those used for captive cetaceans) are not useful for estimating pregnancy rates for wild populations. In captive animals, pregnancy state has been typically monitored either through sonographic visualization of the reproductive tract or the quantification of progesterone or progesterone metabolites found in the blood and urine, which increase dramatically during the onset of pregnancy (Sawyer-Steffan *et al*. 1983; Cornell *et al*. 1987; Walker *et al*. 1988; Duffield *et al*. 1995; Brook *et al*. 2002, 2004). However, due to the innate hazards for researcher and animal, restraining wild cetaceans for ultrasound examination or blood and urine collection are only feasible in the rarest of circumstances. In addition, the concentrations of progesterone often overlap in serum of pregnant and non-pregnant cetaceans (Sawyer-Steffan *et al*. 1983, Temte and Spielvogel 1985, Cornell *et al*. 1987). This is likely caused by the variability of progesterone production by the organ(s) that generate the majority of female reproductive steroids, the corpus luteum and possibly the placenta,[1] resulting in fluctuations of serum progesterone concentration throughout gestation. Although the average concentrations seen in pregnant females are generally much higher than those seen in non-pregnant animals, periods of low production do occur during pregnancy, resulting in concentrations similar to those of non-pregnant animals, especially those levels associated with non-fertile ovulation (*i.e.*, ovulation not leading to conception; Robeck 1996). Consequently, when using solely endocrine markers, a captive female is deemed pregnant only after elevated progesterone levels have been observed in a series of consecutive sampling events (Sawyer-Steffan *et al*. 1983). Researchers studying pregnancy status of wild cetaceans can rarely sample the same animals more than once, and doing this to estimate the pregnancy rate for an entire population would be impractical.

Measuring progesterone concentrations in blubber obtained *via* projectile biopsy procedures may be a practical alternative to analyzing serum or urine for reproductive

[1] To our knowledge, it has not been shown whether the cetacean placenta produces substantial levels of progesterone.

assessment of wild cetaceans. Biopsies are routinely acquired from many cetacean populations to collect tissue to study genetic relationships (Steeves *et al*. 2001, LeDuc *et al*. 2002), sex composition (Escorza-Trevino and Dizon 2000, Gowans *et al*. 2000), diet (Borobia *et al*. 1995, Todd *et al*. 1997, Hooker *et al*. 2001), and contaminate load (Hobbs *et al*. 2003). Researchers using projectile biopsy procedures can non-lethally collect many skin/blubber samples from locations and times of their choosing.

Mammalian adipose tissue, like that found in blubber, is known to accumulate steroid hormones (Dolezel *et al*. 1991), and the concentrations of these hormones have been associated with different pregnancy states (Hamudikuwanda *et al*. 1996). The lipophilic steroids amass in high concentrations in adipose tissue because they can passively diffuse from capillaries into this mostly lipid environment (Deslypere *et al*. 1985, Mead *et al*. 1986), where metabolic and physiological processes that would break down or remove the steroids are slow relative to those occurring in the blood.

Mansour *et al.* (2002) first suggested using projectile biopsy to examine cetacean pregnancy status. The authors, while not actually employing samples collected in this manner, showed that blubber, which is attached to most biopsies, might be used to assess reproductive status. They found that blubber of pregnant minke whales (*Balaenaptera acutorostrata*) killed in Norwegian whaling operations contained, on average, 60 times more progesterone than blubber of immature minkes. However, due to lack of samples, they were unable to compare progesterone concentrations between pregnant and non-pregnant mature females. All of the non-pregnant minkes used in the study had no corpora lutea or corpora albicantia (indicators of maturity in cetaceans), thus leaving the results ambiguous as to whether the high blubber progesterone concentrations were associated with sexual maturation or pregnancy. Given what is known about the dramatic increase in production of progesterone by the corpus luteum during pregnancy, the authors reasonably concluded that endocrine activity during pregnancy was the cause of the higher blubber progesterone levels.

In addition to determining pregnancy status, there are some indications that the blubber attached to biopsies might be used to help determine stage of pregnancy (*i.e.*, the length of time since conception). In all mammals maternal serum progesterone concentrations increase dramatically during the commencement of pregnancy (Bedford *et al*. 1972, Gemmell 1995, Ishwar 1995, Spencer and Bazer 2002). In some of these species progesterone concentrations continue to rise substantially throughout gestation (Bedford *et al*. 1972). In these animals progesterone levels can be used not only to detect pregnancy status but also to roughly estimate stage of pregnancy. Unfortunately, cetacean serum shows no trend in progesterone concentration throughout gestation, at least not substantial enough to help indicate pregnancy stage (Cornell *et al*. 1987, Robeck 1996). Whether this trend is represented in the blubber is unknown.

In our study, to further assess the potential of using projectile biopsies to determine pregnancy status in cetaceans, we extracted and quantified progesterone from blubber samples (similar to those obtained from biopsies) of three species of small delphinids: the short-beaked common dolphin (*Delphinus delphis*), northern right-whale dolphin (*Lissodelphis borealis*), and Pacific white-sided dolphin (*Lagenorhynchus obliquidens*). We examined whether blubber progesterone concentrations distinguish reproductive status in these cetaceans, and we specifically examined whether they accurately differentiate pregnant females from non-pregnant mature ones. We profiled changes in blubber progesterone concentration throughout gestation to determine whether pregnancy stage can be estimated. Finally, we investigated how differences in blubber depth or anatomical location affect progesterone concentration. From these

analyses, we attempted to assess the degree in which progesterone concentrations obtained from blubber can correctly classify pregnancy status.

METHODS

General

This research is composed of two studies: one to validate our technique and the other to examine whether progesterone concentration varies by anatomical sampling site. The validation study examined three delphinid species and compared females of different reproductive states, focusing on the comparison between pregnant and non-pregnant mature females. In addition, we examined whether blubber progesterone concentration changed significantly during gestation to determine whether pregnancy stage could be estimated.

The anatomical sampling study was conducted to determine if progesterone concentration varied at different places on the body, at different depths below the surface of the skin, or both. For the first part of this study a single *L. borealis*, determined to be recently pregnant *via* examination of uterine morphology, was sampled at seven locations, and blubber progesterone concentrations were compared between these locations to determine if the concentrations varied enough to lead to erroneous pregnancy designations. For the second part, progesterone was quantified at three different depths below the surface of the skin in ten pregnant *D. delphis* to determine again if sampling site variability was likely to lead to misclassification of pregnancy status.

Samples

All but one of the blubber samples were taken from dolphins incidentally caught in the California gill net fishery and collected by observers in the California/Oregon Gillnet Observer Program, between 1991 and 2003, following the protocol delineated in Jefferson *et al*. (1994).

The single non-fishery sample, from a recently postpregnant and stranded dead *L. borealis*, was collected by the Southwest Fisheries Science Center Stranding Program. When sampled, this carcass was in the initial stages of decomposition as evidenced by moderate drying and wrinkling of the skin. However, there was no prevalent sloughing or cracking of the skin, and the animal did not appear bloated. In addition, there was no strong decay odor, and the blubber was firm and only slightly blood-tinged.

All fishery samples were collected from the dorsal, mid-thoracic area; data regarding specimen length, girth, and sex were recorded. Pregnancy status, number of corpora, corpus luteum size (where applicable), and length of fetus (if present) were also noted. In summary, we acquired blubber samples from 73 *D. delphis* (18 pregnant, 19 non-pregnant and mature, and 36 immature), 30 *L. borealis* (5 pregnant, 6 non-pregnant and mature, 18 immature, and 1 non-pregnant and mature with distended uterus), and 7 *L. obliquidens* (1 pregnant, 2 non-pregnant and mature, and 4 immature). Samples were stored between 1 and 132 mo in aluminum foil at -20°C before they were extracted. The effect of storage time on measured concentration of progesterone was examined.

Sample Preparation

Cross-sectional subsamples (150 mg; about the amount obtained by a small biopsy), spanning from epidermis to the subcutis distal to the muscle ($\sim$15 mm), were

subsectioned from the large slabs of blubber collected in the field. Care was taken to cut away any areas of discoloration resulting from freezer storage. The thin columns of blubber were then placed into tared homogenization tubes (see below) and weighed. All slabs were subsampled and processed in triplicate.

To examine the variation of progesterone concentrations between different anatomical locations, we sampled the recently postpregnant *L. borealis* specimen at multiple body sites. Blubber slabs, similar to those collected by the observer program, were taken from seven locations along the left side of the specimen. Each slab was then subsampled six times, resulting in a total of 42 subsamples. The subsampling and weighing procedures were the same as described above.

To determine the relationship between blubber depth and progesterone concentration, we examined the outer, middle, and inner layers of the blubber. Subsampling procedures were similar to those described above; however, larger vertical subsamples were taken, each weighing approximately 500 mg. These were then further subdivided into three nearly equal horizontal subsections, each weighing between 100 and 200 mg. These were then weighed in preparation for extraction. Three subsamples were taken from each of the ten pregnant *D. delphis*, for a total of 30 subsamples. These subsamples were further horizontally subdivided, as described previously, for a grand total of 90 vertical sections. From these 90 sections, we examined whether progesterone concentration varied with blubber depth in such a way that misclassification of pregnancy status from biopsies was likely.

In attempts to examine the impact of postmortem decay on progesterone concentration, six subsamples from the blubber of a pregnant *L. borealis* (previously frozen stored at −20°C) were incubated at approximately 22°C for 52 h to approximate the postmortem time of a stranded carcass with minimal signs of decay. Progesterone was measured in these subsamples and compared to concentrations we obtained directly from the frozen blubber.

Steroid Extraction

The subsamples were homogenized in 1,000 μL 100% ethanol using an automated, multitube homogenization instrument (FastPrep Instrument Qbiogene) and were processed for eight 45-s periods at a speed of 6.5 m/s in specialized lysing matrix tubes available from the instrument manufacturer. The homogenates in ethanol were mixed, *via* a multitube vortex mixer (VWR International, VX 2500), and 500 μL were aspirated from each tube and placed into 12 × 100 mm disposable glass culture tubes. The homogenates were centrifuged (3,000 rcf, 10 min), and the supernatants collected. They were evaporated under compressed air using an Evap-O-Rac (Cole-Palmer EW-01610-15) while incubating in 25°C water. Two milliliters of ethanol:acetone (4:1) were added to the residue, vortexed, and centrifuged as before. This solution was evaporated, and to the new residue, 1,000 μL diethyl ether were added. The samples were again vortexed, centrifuged (3,000 rcf, 15 min), and evaporated. To the resulting residue, 1,000 μL acetonitrile was added and thoroughly vortexed. Then hexane (1,000 μL) was added, vortexed, and centrifuged (20 min). The solvents formed two immiscible layers with hexane on top. The acetonitrile layer was collected and re-extracted with 1,000 μL hexane, centrifuged (20 min), and the final acetonitrile layer was aspirated and evaporated. The final residue was centrifuged (2,500 rcf, 5 min) and frozen at −20°C until analyzed.

This extraction method was modified from one delineated in Mansour *et al.* (2002). The modifications were implemented to make the procedure easier and less expensive

to use by reducing the total number of steps, employing an automated multitube homogenization instrument, and substituting compressed air for nitrogen. Twenty samples (eight pregnant, six nonpregnant and mature, and six immature) were extracted four times; twice using our final procedure and twice using the one delineated in Mansour *et al.* (2002). The results of these extractions were examined to assess the comparability of the two procedures.

Enzyme Immunoassay

Prior to analysis, we redissolved all samples in 500 μL of phosphate-buffered saline (pH 7.5) containing 1% bovine γ-globulin and mixed them thoroughly using the multitube vortex mixer at medium speed for 15 min. Progesterone levels were measured using a commercially available enzyme immunoassay kit, DSL-10-3900 (Diagnostic Systems Laboratories, Inc., Webster, TX) with a standard curve range between 0.33 and 1,000 ng/mL. The reported interassay coefficient of variation (COV) ranged from 3.4% to 7.0% and intra-assay COV ranged from 4.1% to 5.0%. Immediately before a sample was added to the assay plate, it was mixed (30 s) using a mini-vortex mixer. To control the measurement error contributed by all extraction and quantification steps, each sample was extracted and measured at least three times and reported as the average nanograms of progesterone per wet weight of subsample.

Extraction efficiency was determined for each group of extractions by spiking selected subsamples with dilutions of cold progesterone, ranging from 0 to 45 ng (45 ng is equal to 300 ng/g for a 150-mg sample and is within the range expected for pregnant females), in the matrix tubes before initial homogenization. We extracted and quantified the progesterone in these subsamples according to the procedure described above. The extraction efficiency was calculated as the amount of quantified progesterone (*via* enzyme immunoassay analysis) of the spiked samples minus the quantified amount in the non-spiked samples, all divided by the original amount of progesterone added (spiked) before extraction. The efficiency range was 63.3%–95.9% with a mean of 71.1%.

Data Analysis and Interpretation

To compare the concentrations of different reproductive classes, we analyzed the data with a one-way ANOVA followed by a *post hoc* Tukey test. The concentrations were log-transformed to reduce heteroscedastic variation from measurement error. An additional one-way ANOVA/Tukey test was conducted with the log-transformed concentrations to test if significant differences in progesterone concentrations were seen between the seven anatomical sampling locations. To assess the relationship between progesterone concentration and gestation time (as estimated by fetus length), a linear correlation analysis was performed with the non-transformed concentrations. Finally, a simple linear regression analysis was employed to assess the effect of storage time on measured progesterone concentration.

To examine progesterone concentration as a function of blubber depth, we obtained average concentrations at each layer (*i.e.*, outer, middle, and inner) in 10 individuals. The significance of the differences in these average concentrations was assessed *via* the Krustal-Wallis test, with the data grouped by layer. This non-parametric test was employed so that females with considerably higher blubber progesterone would not dominate the results.

To assess the difference in concentration in identical samples using the different extraction techniques, a paired *t*-test was employed. Coefficients of variation within and between methods were also determined. A student *t*-test was used to test the significance of differences in the average concentration between samples incubated at ambient temperature and those left frozen.

All statistical comparisons in this study were considered significant at $P \leq 0.05$. Information regarding the depth of sample, anatomical location of the sample, and reproductive status were kept blind to those who were extracting and quantifying the steroids.

RESULTS

Blubber samples from pregnant females had dramatically more progesterone than those from non-pregnant mature and immature females ($P \ll 0.001$ for both comparisons). Pregnant *D. delphis* had, on average, 16 times more blubber progesterone than non-pregnant mature dolphins (Table 1). Moreover, there was no overlap in the range of concentrations of these two reproductive states, but rather a four-fold difference existed between the lowest observed levels in pregnant animals and the highest seen in non-pregnant ones (Fig. 1). No significant difference was seen between progesterone concentrations of immature and non-pregnant mature females ($P = 0.49$). Similar results were seen for both *L. borealis* and *L. obliquidens* (Table 1).

Although a 16-fold difference existed between pregnant and non-pregnant mature animals, no trend in progesterone concentration with stage of pregnancy was observed. Blubber progesterone concentrations taken from 15 pregnant *D. delphis* showed no correlation between progesterone concentration and fetal length ($r = 0.241$, $P =$

Table 1. Progesterone concentrations in the blubber of pregnant, non-pregnant, and immature females of four cetacean species. The progesterone concentrations are corrected for extraction efficiency (see text) and are reported as ng/g of blubber extracted. Average values are displayed with standard error.

Status	*D. delphis*	*L. borealis*[a]	*L. obliquidens*	*B. acutorostrata*[b]
Pregnant				
Average	261 ± 29	312 ± 44	161	132 ± 22
Minimum	132	196	—	22.8
Maximum	415	402	—	454
n	18	5	1	22
Non-pregnant/mature				
Average	13.7 ± 1.8	15.0 ± 7.5	12.1 ± 8.4	Not available
Minimum	6.75	2.11	3.75	
Maximum	33.3	34.7	20.5	
n	19	6	2	
Immature				
Average	16.5 ± 2.7	14.2 ± 2.30	18.1 ± 9.1	1.95 ± 0.32
Minimum	0.92	0.98	0.11	1.36
Maximum	48.2	33.1	34.4	3.43
n	36	18	4	6

[a]Does not include the *L. borealis* sample with distended uterus.
[b]From Mansour *et al.* 2002.

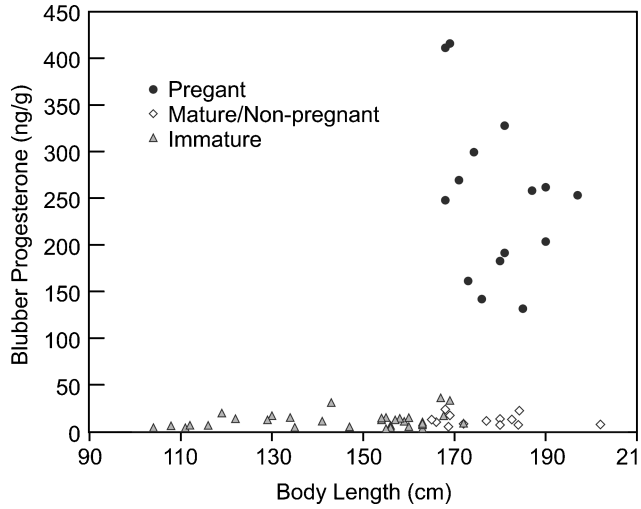

Figure 1. Blubber progesterone concentrations as a function of total body length in pregnant, mature but not pregnant, and immature female *D. delphis*.

0.387; Fig. 2). However, we note that the sample set contained only one fetus larger than 40 cm, which is approximately the halfway point of fetal development in this species. Given this sample distribution, an increase or decrease in concentration during the second half of gestation could have been missed. Too few specimens were collected from the other species to conduct a meaningful correlation analysis.

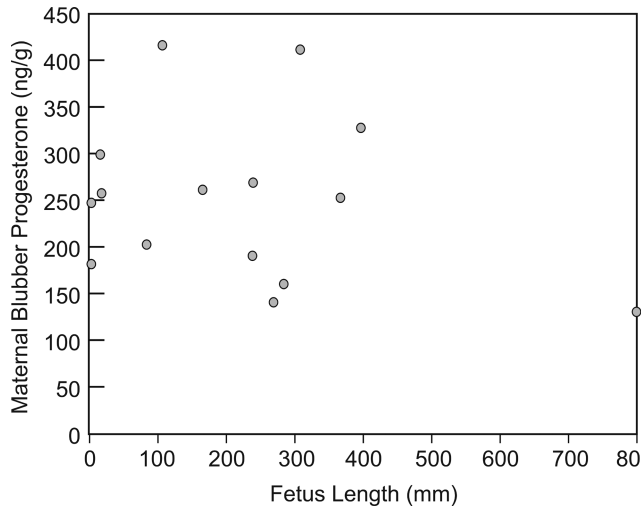

Figure 2. Maternal blubber progesterone concentrations as a function of fetal length of 15 *D. delphis*. The estimated fetal size at parturition between 800 and 900 mm (Perrin 2002).

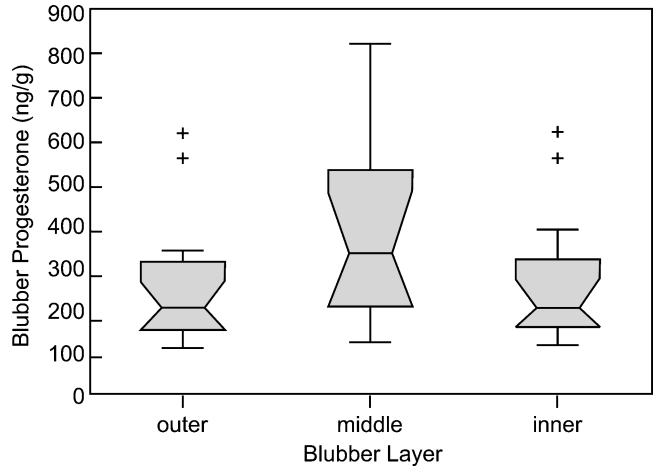

Figure 3. Blubber progesterone concentrations for outer, middle, and inner blubber layers. The concentrations were quantified from ten pregnant *D. delphis*. Horizontal box lines represent the lower quartile, median, and upper quartile values. Whiskers lines indicate range of concentrations and the pluses represent outliers (1.5 times interquartile range). Points of inflection represent upper bound to the 95% confidence interval. The lower CI bounds were not shown as they were lower than the first quartile line. No significant differences were found between any of the layers.

Average blubber progesterone concentrations were relatively similar at different depths below the skin (Fig. 3). Although concentrations from the middle layer were on average 1.45 and 1.39 times higher than in the outer and inner layer respectively, we did not find significant differences between the three depths ($\chi^2 = 3.16$, $P = 0.21$). More importantly, there were no substantial differences found between layers; they were not at the same magnitude as the difference observed between pregnancy states. As a result, where in the blubber layer a subsample was obtained would not cause a misclassification, because the concentrations at any layer were still within the range found for pregnant animals.

The blubber samples from the recently postpregnant *L. borealis* yielded two important findings. First, though there were significant differences between the peduncle sample and three of the more anterior samples ($F = 4.37$, $P = 0.0026$; Fig. 4), they were not substantial. The variation of blubber progesterone concentration due to anatomical location should not compromise the accuracy of pregnancy designation. The lowest concentration (40.9 ng/g in the peduncle) was still 57.8% of the highest concentration (70.8 ng/g in the dorsal midline, posterior to the flipper). When compared to the results shown in Table 1, where the highest concentration in the non-pregnant *L. borealis* females (34.7 ng/g) is only 17.7% of the lowest concentration in the pregnant ones (195 ng/g), we found that the variation due to body location was diagnostically small. Second, we found that blubber progesterone concentrations of postpregnant *L. borealis* were higher than the levels we saw in non-pregnant females but still much lower than those seen in pregnant females. This was the only value in our sample set where the levels were between those seen in pregnant animals and those seen in non-pregnant animals, and the only one that could have been misclassified.

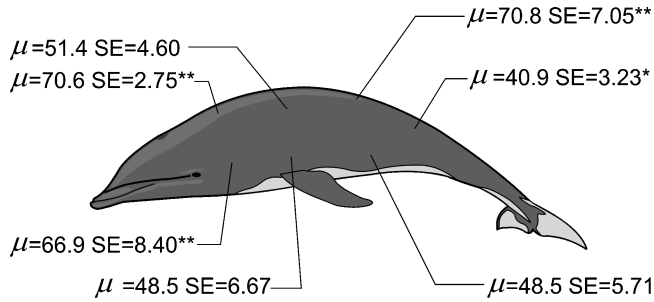

Figure 4. Blubber progesterone concentrations at seven anatomical locations in a recently pregnant *L. borealis*. Six subsamples were quantified at each location. Though there are significant differences between concentrations found at the peduncle sampling location (*) and three of the more anterior sampling areas (**), these differences were relatively small compared to those found between pregnant and non-pregnant animals (see text).

We found no significant relationship between storage time at −20°C and measured progesterone concentration in *D. delphis* blubber samples. This finding was consistent across the three reproductive groups: pregnant ($r = 0.310$, $P = 0.260$), non-pregnant mature ($r = 0.411$, $P = 0.169$), and immature ($r = 0.153$, $P = 0.375$).

Though the six samples incubated at ambient temperature for 52 h exhibited signs of decay (discolored tissue and pronounced odor), we found no significant differences between their average progesterone concentrations and those from the frozen samples ($t = 0.617$, $P = 0.551$), suggesting that progesterone does not decrease rapidly in blubber samples kept at ambient temperature. In fact, average concentrations were slightly higher in the incubated samples (218.6 ng/g *vs.* 199.7 ng/g).

When comparing the two extraction procedures (ours and the one described in Mansour *et al*. 2002), we found no significant differences in the concentrations using paired *t*-tests for each of the reproductive groups (pregnant $P = 0.23$, non-pregnant and mature $P = 0.56$, and immature $P = 0.44$). We also found that the variability between extractions of identical samples using the same procedure was as high as the variability between procedures. The average paired coefficients of variation (COV) were 19.9% for the Mansour *et al.* procedure and 18.1% for the one delineated in this paper. Between procedures, the COV value was 19.6%. These results suggest that the data derived from the two procedures are comparable; however, they also show that variation is high using either procedure. Therefore, replicate extractions are recommended when specific concentration is desired. Nonetheless, this amount of variation is small when compared to the differences in average concentration between pregnancy states in these delphinids and therefore is not problematic for pregnancy determination.

Discussion

Although progesterone has been previously extracted and quantified from cetacean blubber (Mansour *et al*. 2002), the data in this study are the first from delphinids and the first that show differences between pregnant and non-pregnant mature cetaceans, clearly a crucial distinction when detecting pregnancy. All but one of the female cetaceans ($n = 110$) examined in this study could be assigned to their correct reproductive status based solely on blubber progesterone concentration. In addition,

this study indicates that potential sources of misclassification due to the vagaries of projectile biopsy sampling are unlikely to affect pregnancy determination. Thus, we are confident that blubber progesterone is an accurate indicator of cetacean pregnancy in wild populations. This is fortunate because there are currently no other practical alternatives except in very circumscribed situations.

The blubber progesterone levels of pregnant and immature animals found in the three species examined in this study were higher than those found in minke whales (Mansour *et al*. 2002). However, in both studies, pregnant animals were found to have much more blubber progesterone than immature animals; 60-fold more in minke whales and 16-fold more in the three species of delphinids studied here. Similarly, we found that blubber progesterone concentrations in pregnant females were much higher (19 times) than those found in non-pregnant mature animals. For our subjects, this indicates that higher progesterone levels are, as expected, associated with pregnancy and not maturity.

The differences in blubber progesterone concentration that distinguish pregnant and non-pregnant animals can be contrasted with the differences found by examining serum and urine samples. Although elevated levels of progesterone in serum and progesterone metabolites in urine have previously been used as indicators of pregnancy in *Tursiops truncatus* (Cornell *et al*. 1987), *Orcinus orca* (Duffield *et al*. 1995), *Balaenoptera physalus* (Olafsson and Kjeld 1986), and *Phocoenoides dalli* (Temte and Spielvogel 1985), the differences between pregnant and non-pregnant concentrations of progesterone and progesterone metabolites overlap substantially (Sawyer-Steffan *et al*. 1983, Robeck 1996). In contrast, blubber progesterone concentrations from pregnant and non-pregnant cetaceans did not overlap in this study or the one conducted by Mansour *et al*. (2002). The only specimen (the *L. borealis* that was sampled in seven anatomical locations) that fell between the levels observed for pregnant and non-pregnant animals was a recently postpregnant female transitioning between reproductive states (discussed below).

Although blubber progesterone concentration seemed to be an accurate indicator of pregnancy status, it did not provide information to differentiate stage of pregnancy in the specimens of *D. delphis* examined here. Instead, as in cetacean serum (Cornell *et al*. 1987), the level of progesterone in blubber showed no clear trend with time in gestation.

Blubber progesterone concentrations obviously must track serum concentrations, albeit lagging in some complex manner. In other cetartiodactyls, strong correlations between serum and subcutaneous adipose tissue have been observed (Hillbrand and Elsaesser 1983, Hamudikuwanda *et al*. 1996), although adipose concentrations have been found to lag behind those in serum by about 16–24 h when progesterone levels are increasing in the serum and 32–50 h when levels are decreasing (Hillbrand and Elsaesser 1983). More research is required to elucidate the specifics of the dynamic relationship of progesterone concentrations in cetacean blood and blubber, but the data obtained in this study are in agreement with the common wisdom that they are tightly associated.

Important for sampling *via* projectile biopsy, we observed that blubber progesterone concentration did not substantially change with anatomical location or depth of sampling. This is in contrast to other non-polar macromolecules, such as naturally occurring triglycerides, which are generally thought to be strongly stratified in both spatial distribution, concentration, and identity (Aguilar and Borrell 1990, Olsen and Grahl-Nielsen 2003). Differences in these relative concentration distributions may be due to differences in intercellular transportation of the lipophilic macromolecules.

Steroids like progesterone are thought to diffuse passively from the blood into adipose tissue with minimal metabolic processing (Hillbrand and Elsaesser 1983, Mead *et al*. 1986). In contrast, most triglycerides must be broken into their basic metabolic units (*i.e.*, glycerols and fatty acids) before they can be transported through cell membranes (Mead *et al*. 1986), possibly leading to the regulation of triglyceride distribution in the blubber.

Although sampling location does not appear to affect pregnancy determination, there are several situations not addressed in this study that could potentially lead to misclassification. Corpora lutea that are associated with non-fertile (*i.e.*, not leading to conception) estrous cycles also elevate serum progesterone concentrations. However, these levels are usually lower than the average concentrations seen in pregnant animals (Brook *et al*. 2004). Because we did not obtain samples from females in this state, we were unable to document its effects on blubber progesterone concentrations. A similar situation arises with pseudo-pregnant females. In this condition, the reproductive tract behaves as though it were pregnant; the corpus luteum is maintained and produces progesterone concentrations in the serum at levels similar to those seen during pregnancy (Robeck *et al*. 2001). Although we saw no evidence of this condition in the samples we examined, it is observed in captive animals, and if present in wild cetaceans, it would likely produce misclassifications of status. Both conditions, non-fertile estrous cycles and pseudo-pregnancies, would lead to over-estimates of pregnancy rates in wild populations based on blubber progesterone levels. The rates of these conditions are unknown, and as such, their potential bias cannot be precisely estimated.

Another potential source of error in utilizing blubber progesterone levels to determine pregnancy, is the timing of the elevation and decline in blubber progesterone with respect to commencement and termination of pregnancy. In our study, we observed that in embryos with total lengths <1 cm or approximately 2–3 wk post-conception (Šterba *et al*. 2000), blubber progesterone levels were between 183 and 248 ng/g, well within the total range we see for females with larger fetuses and much higher than those found in non-pregnant animals. This suggests that progesterone levels rise quickly in the blubber at the commencement of pregnancy. Clearly, at some earlier point during gestation, blubber progesterone concentrations are not effective at distinguishing pregnancy status. We suspect that the blubber progesterone levels rise to moderate levels post-estrus, as they do in the blood, and then rise further after implantation. Thus, the concentrations seen during the very earliest stages of pregnancy would likely overlap with the levels seen immediately after non-fertile estrus.

Similarly, after parturition, when progesterone levels are declining, it is likely that there is a short period when progesterone levels would be ambiguous. Females in this state, though no longer pregnant, are likely to have blubber progesterone concentrations that are still elevated. How long blubber progesterone remains elevated is unknown. However, we do know that the beach-cast, recently post-pregnant *L. borealis* had a distended uterus, indicating that it had been pregnant within the few days before its stranding. We do not know exactly how long after the termination of its pregnancy the animal was sampled. Nevertheless, this female's progesterone levels were already down to 30% of the lowest level we observed for *L. borealis* carrying a fetus. In most mammals, after normal parturition, the uterus regains prepregnancy state within a short period (Henell *et al*. 1983, Ouellette and Ronald 1985 Katila 1988, Dolezel *et al*. 1991). For example, in cattle, uterine morphology returns to non-gravid shape within ten days postparturition (Dolezel *et al*. 1991). Given this short

physiological window in which the uterus is distended and the dramatically lower blubber progesterone levels found in the postpregnant *L. borealis*, it is likely that the decrease in progesterone occurs rapidly after parturition. Knowing the precise relationship between apparent pregnancy (*i.e.*, elevated levels of blubber progesterone) and true pregnancy (*i.e.*, the presence of an embryo/fetus) would allow us to more accurately estimate pregnancy rates in the wild.

Another plausible explanation for the lower levels of progesterone found in this animal is that the processes involved in decay reduced the levels of progesterone as a proportion of tissue mass. In attempts to address this issue, we incubated blubber subsamples at temperatures roughly equal to those that a carcass might be exposed to before it could be sampled. We found no significant differences between their average progesterone concentrations and those from the frozen samples. In fact, average concentrations were slightly higher in the incubated samples (218.6 ng/g *vs.* 199.7 ng/g). This suggests that progesterone does not decrease rapidly in blubber under "ambient" conditions. This finding is consistent with other studies that show progesterone can be stable in various biological samples at temperatures above 20°C (Wiseman *et al.* 1983, Eissa *et al.* 1995, Groschl *et al.* 2001, Galama *et al.* 2004).

Although there are several potential sources of error inherent in determining pregnancy status *via* blubber progesterone concentrations, to our knowledge there are no other practical means to obtain non-lethal estimations of pregnancy rates in wild cetacean populations. Given the importance of determining these rates for population dynamic models and the lack of other means to do this, we believe that this approach, when coupled with projectile procedures, will be a useful tool for researchers.

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